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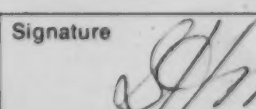

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EARLY CHANGES IN THE GALLBLADDER EPITHELIUM IN RESPONSE TO EXPERIMENTAL INDUCTION OF GALLSTONES IN RABBITS

by

SULE GARBA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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A B S T R A C T

This study investigated early structural changes in the gallbladder in response to the ingestion of a lithogenic diet.

Rabbits were fed a diet containing 1% w/v of dihydrocholesterol (DHC) and control animals were fed the normal rabbit pellet. The gallbladder was removed from experimental animals at daily intervals up to five days following the ingestion of the diet. The size of the organ was determined by volume of water displacement. Histologic and ultrastructural studies were made with light and electron microscope respectively, and mucus glycoprotein was determined histochemically.

DHC-rich diet caused no increase in the size of the gallbladder. Within 24 hrs the production of mucus was increased and osmiophilic lipid material appeared intercellularly as well as within foam cells in the lamina propria. Early intracellular epithelial alterations involved dilation of both the rough and smooth endoplasmic reticulum and mitochondria. Cells exhibiting these intracellular changes also showed distortion of the apical plasma membrane including microvillous pattern and configuration. Progressive focal epithelial disruption and release of cellular debris into the gallbladder lumen followed.

It is concluded that the gallbladder epithelium is injured and mucus secretion increased very early in this model of experimental cholelithiasis. There is no distention of the gallbladder and intramural deposition of osmicated lipid droplets occurs in this model of gallstone formation.

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INTRODUCTION: Perspective on gallstone disease

Gallstone disease is a major, and increasing cause of morbidity, admission to hospital and burgeoning health costs, in industrialized countries. Of the total Canadian population of approximately 24 million persons (1), each year about 130,000 are admitted to hospital because of this disease and 80,000 of them ultimately undergo cholecystectomy (2). In monetary terms, the estimated annual cost of gallstone-associated morbidity is 280 million dollars in Canada (2) and one billion dollars in the U.S.A. (3).

Most of the current statistics on this disease are based on hospital data chiefly of symptomatic cases and necropsy findings, both of which underestimate the true prevalence in the population (2-4). In the U.S.A., a recent review of the epidemiology of cholelithiasis (3) indicated that about 16 million people have gallstones, that each year about 800,000 new cases are diagnosed, half of these patients ultimately requiring surgery, and 5,000 to 8,000 deaths are due to the disease each year. The problem is much greater in some North American Indian tribes; studies in the Pima in the U.S.A. (5) and Micmacs in Canada (6) have shown that approximately 70% of the women have gallstones by about 30 years of age and a similar frequency in the Pima men in a much later age.

Epidemiological reports from other parts of the world have shown high rates of the disease in Europe, especially Sweden, but fewer cholecystectomies than in Canada and the U.S.A.(2,7). The incidence of

cholelithiasis is increasing in Japan (8), but is still rare in the Masai, a mainly nomadic East African tribe, and in Canadian Inuit (2,3).

Several authors have postulated a positive correlation between gallstone disease and such factors as age, sex, social class, genetics, obesity and diet, and diseases such as pancreatitis, diabetes mellitus, atherosclerosis, and hyperlipidemia (3). It is much commoner in females than in males. Cholelithiasis is rare in childhood, but the frequency increases with age in both sexes (3). Recently the disease has been reported in premature infants with respiratory distress syndrome and bronchopulmonary dysplasia who were being treated with assisted ventilation, total parenteral nutrition and furosemide (9).

Gallstones are classified by their cholesterol content (pure or mixed) or as pigment stones. In the U.S.A., 80% of stones are the mixed variety (10), containing at least 70% cholesterol by weight; bile pigments and acids, calcium salts, and protein constitute the remaining 30%. It is not known whether the non-cholesterol particles are trapped by cholesterol crystals as they grow or are active participants in stone formation. Pigment stones usually occur in association with chronic hemolytic disorders and liver cirrhosis (7).

Despite the recent advances in knowledge of biliary-tract physiology and the physiochemical properties of bile, the pathogenesis and natural history of gallstone disease remain unresolved. This is partly because the study of cholelithiasis has lagged behind most areas of medical research (4,11,12). Gallstones may produce symptoms with or without associated pathologic and functional changes, or may produce no symptoms but be detected on abdominal roentgenograms taken for other reasons or at necropsy (4). The disease tends to run a benign course in

the early stages, becoming symptomatic only when the stones have become large enough to block the cystic duct or induce cholecystitis (7). Also, small stones passing through the common bile duct produce pain and acute pancreatitis.

Neoplastic degeneration of the gallbladder has been reported in a small proportion of people with long-standing cholelithiasis (13,14). Gallstones may relate to development of carcinoma of the gallbladder: 1) gallstones are found in about 75% of gallbladders removed at operation for this tumor and in about 90% of cases in which it is found at necropsy (14) and 2) the tumor is commoner in females than in males - a pattern similar to that of gallstone disease (14). Adenocarcinoma is the commonest histologic type; sarcoma (14,15) and pleomorphic spindle-cell carcinoma (16) are very rare.

At present, cholecystectomy is the definitive treatment of gallstone disease; medical dissolution of the stones is still experimental (10). The operation carries a very small but definite risk and is inadvisable in very few patients (13). Thus the problem of gallstone disease is likely to remain until the pathogenesis and natural history have been clearly defined and pharmacologic agents or other measures such as dietary regime have been developed to prevent or treat the disease.

REVIEW OF THE LITERATURE

Morphology of the Gallbladder

GROSS ANATOMY

There are no major differences in the gross anatomy of human and rabbit gallbladder (17). This oblong, sacculated organ, commonly described as pear-shaped, is situated in the fossa on the inferior surface of the right lobe of the liver. Peritoneum covers all of its surface except where the organ is in contact with the liver. It consists of three parts: the fundus, body, and neck. In humans, in the erect posture, the body of the gallbladder passes upward and backward into the neck; thus the fundus becomes the most dependant part, rendering gravity ineffective in emptying the organ (7). A bulge (Hartman's pouch) in the neck region, which used to be considered a normal anatomic finding, is now regarded as the result of a pathologic process (18). Congenital anomalies can result in abnormal number, size, shape and location of the organ (19).

The arterial blood supply to the gallbladder comes from the cystic artery, which usually arises from the right hepatic artery. There are many variations of this pattern: and only about 50-60% of cases conform to the 'usual' arrangement; in 20%, accessory arteries are present, and in another 20% the cystic artery is replaced by other vessels (18). Most of the venous drainage is by small veins; most of these empty into the liver, and others drain into the cystic branch of the portal vein.

The rich lymphatic system of the organ begins in two main plexuses, one in the lamina propria and the other in the subserosal connective tissue; vessels that arise on the left side of the organ drain into the cystic lymph nodes, and those from the right side drain into the superior pancreaticoduodenal nodes (18). The organ is supplied by both parasympathetic and sympathetic nerves; a myenteric plexus exists in the lamina propria and subserosa.

HISTOLOGY

The wall of the gallbladder consists of four layers:

1. an epithelial lining
2. connective tissue layer (lamina propria)
3. a fibromuscular layer
4. a serosal layer

The mucosa is in folds of various heights and widths, which are diminished when the organ is distended. In the human gallbladder, crypts between the folds project into the lamina propria; in cross section they resemble tubular glands. 'True' glands are rare in the fundus and body of the gallbladder but are present in the neck region.

1.Epithelial lining. There are some species variations. In all species examined, the lining contains a single layer of tall columnar cells which have pale-staining cytoplasm and basally located nuclei. Light microscopy reveals a faint brush border on the luminal aspect of the cells. In human gallbladders the lining consists predominantly of tall cells with rod-shaped ('pencil') cells, and basal cells also occuring (20,21). The 'pencil' cells are found mainly in the fundus and, like the columnar cells, they extend from the basement membrane to

the lumen, bearing microvilli on their apical border. They appear compressed from the sides, and their darker-staining cytoplasm distinguishes them from the light-staining columnar cells. Basal cells appear much darker than their neighbors. Although each basal cell rests on the basement membrane, all remain wedged between the basal aspect of adjacent columnar cells and have no contact with the lumen. In humans, goblet cells are absent from normal adult gallbladder epithelium but cup-shaped cells presumed to be goblet cells are present in the 7 month fetus (22). In sheep, but not rabbits, goblet cells are present (23).

2.The Lamina propria. This is a loose connective tissue layer containing many blood and lymphatic vesels. In human gallbladder, but not in the rabbit, the lamina propria contains simple tubulo-alveolar glands in the neck region (24); they are lined by cuboidal cells which have basally situated nuclei and secrete mucus. These glands have often been confused with Rokitansky-Aschoff sinuses; the latter are mucosal pseudodiverticula, which also are lined by cuboidal epithelium but penetrate the muscle layer of the wall. They are considered evidence of a pathologic process that has weakened the gallbladder wall, allowing invagination of the mucosa (24).

3.The fibromuscular layer consists of an irregular meshwork of longitudinal, transverse, and oblique smooth-muscle fibers interpersed with collagenous, reticular, and elastic fibers.

4.The serosal layer is composed of flattened mesothelial cells. In some human gallbladders, small aberrant bile ducts (Luschka's ducts) are present in the subserosal layer.

ULTRASTRUCTURE OF THE GALLBLADDER

The most conspicuous feature of the gallbladder's surface morphology is the mucosal folds, which are particularly well visualized by scanning electron microscopy (EM). These folds are due mainly to upheavals of the connective-tissue layer. The epithelial lining follows these outlines; in addition, it folds independently, producing minor creases (25). Mueller *et al.* (26), in studies of guinea-pig gallbladder, showed that this morphologic picture relates to the degree of distension of the organ. When the gallbladder is full, the only evidence of mucosal folds consists of sparse low ridges that overlie large submucosal blood vessels. Flattening of the folds reveals the mouths of crypt-like glands and secretions discharging from them. In guinea pigs, these glands (which are absent from rabbit gallbladder (17)) are located all over the mucosa but are more concentrated in the ductal end. Their lining cells do not differ from the other epithelial cells; histochemically that they contain PAS-reactive material, possibly glycoproteins, in the apical cytoplasm.

Whether these mucosal folds are physiological units is not known. In a combined physiological and ultrastructural study of rabbit gallbladder, Kaye *et al.* (17) found that, in specimens transporting fluid, dilation of the lateral intercellular space was limited to the upper part of these folds. They therefore postulated that absorptive function was restricted to the tips of the folds. In other studies of rabbit gallbladder epithelium (27), autoradiography *in vivo* and *in vitro* revealed greatest mitotic activity in the valleys between the folds. This led to the suggestion that the valleys might represent the site of cell renewal of gallbladder epithelium. However, this concept has been

challenged by the findings in studies of guinea-pig gallbladder, that mucosal folds were present only when the organ was unfilled (26) and that mitotic activity was not restricted to any one part of the mucosa (28).

The appearance of the epithelium on transmission electron microscopy (TEM) of human (20), rabbit (17,29), and guinea-pig gallbladder (26) shows some minor variations. The folded mucosa is made up of a sheet of tall columnar cells that are separated from the underlying lamina propria by a basement membrane. The most striking feature of the apical plasma membrane consists in fairly well-developed microvilli coated with PAS-positive material, probably mucopolysaccharide (29,30). The nature of membrane-bound vesicles and granules near the apical surface is still a subject of discussion. The earliest hypothesis held that they represent pinocytotic vesicles (31,32); pinocytotic uptake of particles in rabbit gallbladder was demonstrated in one study (17) but not in another (29). However, recent studies on human and animal gallbladders have shown that these granules tend to fuse with the apical plasma membrane, and are discharged into the lumen by exocytosis (22,33).

Wahlin *et al.* (34) injected Thorotrast into mouse gallbladder *in vivo* reported pinocytotic uptake of particles of thorium oxide by epithelial cells. The particles were seen in membrane-bound apical vesicles but none was observed in the apical and subapical granules. Feeding olive oil to the mice resulted in free discharge of the subapical granules from the cell surface to the lumen. It was concluded that gallbladder epithelium is capable of pinocytosis but that this is not essential for bile concentration (23), and that the apical and

subapical granules are secretory (34). There have also been reports of the occasional protrusion of cytoplasm into the gallbladder lumen in fetal rabbits (35), humans (36), dogs (37), rainbow trout, and tench (38). The protruded cytoplasm is membrane-bound and free of granules or organelles, a finding whose significance is unknown.

Like the luminal border, the lateral cell surface of adjacent cells has a striking appearance on TEM. This feature has been well illustrated, by Kaye et al. (17) and Tormey et al. (29) with rabbit gallbladder, and has been described in many other species, including man (20), but is absent from fetal rabbit gallbladder epithelium (35). The apical ends are fused by tight junctions and appear more electron-dense than lower regions. The cell surface is straight from below this tight junction down to the level of the nucleus where it erupts into microfolds that interdigitate with similar folds protruding from adjacent cells. The degree of interdigitation depends on the cell's functional state (17,29): in cells engaged in fluid transport, the lateral intercellular space is dilated and the interlocking is loose. In the basal regions, these folds are not well developed, therefore, the lateral intercellular space communicates with the basement membrane and the underlying lamina propria but not with the lumen (29).

The cytoplasm can be roughly divided into five zones (34): apical, subcuticular ('dark'), supranuclear, nuclear, and basal. The apical zone is clearly apparent in human (20, 21) and rabbit gallbladder (17, 23, 29). It is devoid of organelles, and contains filamentous structures that converge on a terminal web (29). In general, the cytoplasmic organelles display only one striking feature - the mitochondria, which appear as electron-dense structures with a double limiting membrane and

few transverse cristae (23). The mitochondria are widely distributed throughout the cytoplasm, excluding only the apical zone; they are most numerous in the 'dark' zone. The granular endoplasmic reticulum is well-defined, and the supranuclear Golgi apparatus (which in the mouse is responsible for mucus-secretion) is well developed (39).

Functions of the Gallbladder

The gallbladder performs two main functions:

1. It collects and stores bile between meals.
2. It delivers the stored bile to the intestine during meals, to facilitate the absorption of fat (40).

The importance of this organ appears to lie not so much in its function but in its being the site of a very common disease. Removal of the gallbladder has very little effect on the body's capacity to handle dietary fat, although steatorrhea has been reported in a few patients (40). Nevertheless, it is reasonable to assume that the ability to deliver large quantitites of bile salt into the intestine is of benefit for those species whose members eat large fatty meals at infrequent intervals (41).

Bile is formed in the interhepatocyte bile canaliculi, secreted into the bile ducts, and is modified there by the absorption and secretion of water and electrolytes (40). It contains lipid, bilirubin, and electrolytes; the three main constituents (42) are bile salts (72%), phospholipids (12%), and cholesterol (4%). Bile salts are synthesized from cholesterol, a process that is controlled by a negative feed-back mechanism whereby the rate of synthesis varies inversely with the amount

of bile salts returning to the liver from the intestine (40, 42). Bile salts tend to aggregate to form micellar complexes with cholesterol and phospholipids. In this process, the lipophilic portion of each of the three molecules is directed toward the center of the complex and the hydrophilic portions of the bile salts are oriented peripherally, thus rendering the micelle soluble in an aqueous environment. Consequently, the solubility of cholesterol depends upon the amount of bile salt and phospholipid available for micelle formation (40, 42).

Bile is produced continuously by the liver, and during fasting periods about 75% gets diverted to the gallbladder (43). With a capacity of about 40-50 ml (40, 44) the organ can store this quantity of bile by selectively absorbing some electrolytes and water (41). Sodium and chloride ions are probably transported into the cell by a carrier molecule situated at the luminal cell surface (42). Sodium ions are pumped out of the cell into the lateral intercellular space by the sodium:potassium pump located at the lateral cell membranes, and chloride ions drift out to join the sodium ions. The absorption of bicarbonate ions is probably effected by the chloride/sodium carrier system or by a separate bicarbonate/sodium carrier system(42). Active transport of ions from the cell into the lateral intercellular space creates an osmotic gradient; this moves water from bile, through the cell, into the intercellular space, until osmotic equilibrium is reached (17). Loss of water greatly increases the concentration of bile salts and unabsorbed lipids.

Stored bile is delivered to the duodenum by the simultaneous contraction of the gallbladder musculature and relaxation of the sphincter of Oddi. Gallbladder motility is regulated by a complex

interaction of the intrinsic myenteric neural plexus, vagal and sympathetic external innervation and hormones. The primary determinant of gallbladder motility after meals is cholecystokinin, which is released by mucosal cells in the upper part of the small intestine in response to specific nutrients - mainly fatty acids (44).

The Gallbladder and the Evolution of Cholesterol Gallstones

The current concept of the pathogenesis of cholesterol gallstones centers on the production of a cholesterol-supersaturated bile (45-47), participation by the gallbladder in the nucleation and precipitation of excess cholesterol into microcrystals, and the growth of these crystals into macroscopic stones (48-50).The classic work of Admirand and Small, reported in 1968 (45) demonstrated that patients who have cholesterol gallstones produce lithogenic bile containing too much cholesterol and not enough bile salts or phospholipids for micelle formation. Accordingly, a lot of the research since then has sought to determine conditions under which bile becomes supersaturated with cholesterol (i.e. lithogenic).

Some authors postulated genetic or metabolic defects within the hepatocytes as being responsible for the production of this abnormal bile (51), a concept that regarded cholelithiasis as a consequence of disordered hepatic metabolism and assigned a passive role to the gallbladder. Later studies identified the liver as the source of the lithogenic bile (52). However, no metabolic or genetic factors have been delineated and no functional or anatomic defects have been found

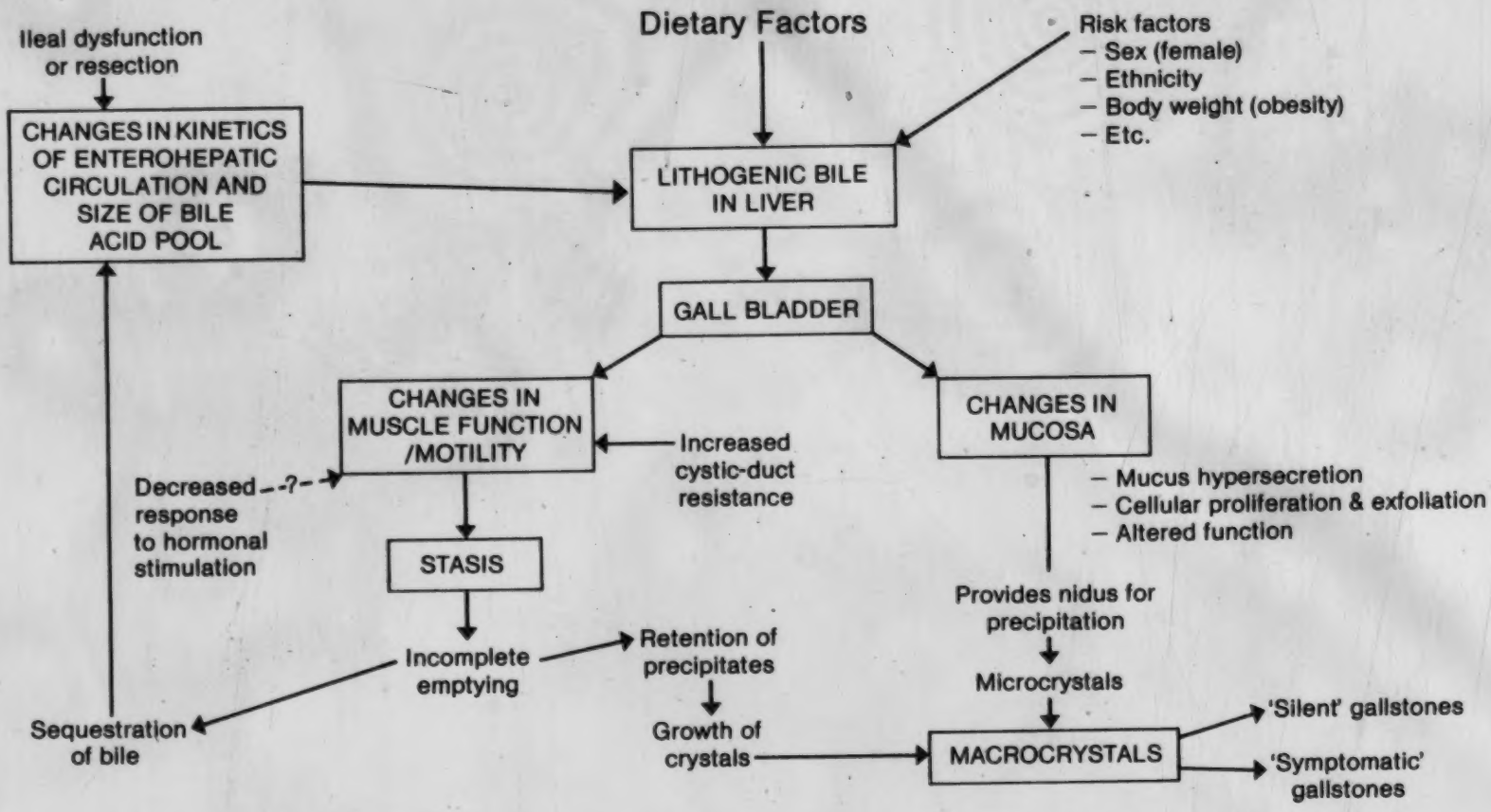
associated with the liver in gallstone disease (52).

Although lithogenic bile is necessary to the formation of cholesterol gallstones, this is not sufficient cause by itself for the precipitation and aggregation of stones (53, 54). Bile from people who produced lithogenic bile but do not form stones crystallized after incubation for 16 days at 37°C, whereas bile from patients who had gallstones formed crystals after only 3 days (53). These data suggest that antinucleating factors that solubilize the excess cholesterol, are present in the supersaturated bile of persons without stones (54) or that nucleating factors exist in the bile of gallstone patients (50). Cholesterol gallstones rarely form beyond the gallbladder, and new ones rarely form in the bile duct after cholecystectomy - even if the production of lithogenic bile continues (55). Thus, as the gallbladder appears to provide a suitable milieu for stone formation, one may reasonably assume it is the site of the additional factor(s) necessary for the nucleation and precipitation of cholesterol crystals and for their retention and growth to stones.

Many aspects of the probably complex role of the gallbladder in the pathogenesis of cholesterol gallstones are unclear. Figure 1 outlines the probable stages of cholesterol gallstone formation. For the purposes of this review, these can be considered under two main headings: 1) muscular (changes in motility) and 2) mucosal.

CHANGES IN MOTILITY

Evidence supporting the role of gallbladder stasis in the pathogenesis of gallstones has come from both clinical and laboratory observations (49). Increased incidence of gallstones has been reported



SCHEME OF FORMATION OF CHOLESTEROL GALLSTONES. FIG. 1

in patients who have undergone truncal vagotomy, which interrupts the parasympathetic nerve supply to the gallbladder and thus leads to dilation and decreased contractility (56). The induction of biliary stasis by part or complete obstruction of the cystic duct led to stone formation in rabbits and dogs (57). In cholesterol-fed prairie dogs, stasis developed (58) and cystic-duct resistance increased (59) during lithogenesis, before stones had formed, and abolishing the stasis by sphincterectomy (60) or by giving physiological doses of cholecystokinin (61) prevented the formation of gallstones. Gallbladder stasis enhances the retention of microcrystals and their growth into macroscopic stones (7). Furthermore, stasis-induced incomplete emptying of the gallbladder can lead to interruption of the enterohepatic circulation, further increasing the production of lithogenic bile (10) [Fig. 1].

However, the factors that initiate stasis in these patients and animal models have not been identified. In studies of cholesterol-fed prairie-dogs, it is speculated that the lithogenic bile might irritate the gallbladder wall, inflaming it and leading to narrowing of the cystic duct (59). Other contributing factors may be increased viscosity of bile, due to mucus hypersecretion (50) - which also precedes stone formation - or alteration by lithogenic bile of the gallbladder/cystic-duct complex's response to hormonal stimulation. The latter is supported by the preliminary results of studies on prairie dogs, which showed impaired gallbladder response to the intravenous injection of cholecystokinin during lithogenesis (67).

Although gallbladder stasis is not sufficent cause <u>per se</u> for the nucleation and precipitation of excess cholesterol out of lithogenic bile (62,63), it does appear to potentiate the process in its early

stages or to induce the appropriate conditions for its initiation. Consequently, attention is now being focused on changes in the gallbladder epithelium during lithogenesis as a possible source of nucleating agents (50, 64).

MUCOSAL CHANGES

Bacterial inflammation of the gallbladder wall is one of the oldest theories put forward to explain the pathogenesis of gallstone formation (49, 65). According to this concept, inflammation causes the exfoliation of epithelial cells, which then provide a nidus for cholesterol crystallization. Infection of the gallbladder probably plays a key role in the genesis of biliary calculi in India (66) and did so in Japan before the adoption of Western culture there (63). The stones are composed primarily of calcium-bilirubinate and some protein. Early attempts at producing animal models of cholelithiasis relied on infecting the gallbladder and its contents with bacteria (67). However, clinical and laboratory studies in Western countries have ruled out an infective pathogenesis in cholesterol cholelithiasis (68), having demonstrated no bacterial growth on culture of human bile in a significantly high proportion of cases. When present, bacterial infection is considered secondary; the commonest pathogens are E. coli, Strep. faecalis, and staphylococci (68).

Another view is that inflammation of the gallbladder wall is probably the result of chemical irritation (68-70). Mosbach and Bevans (69) who fed dihydrocholesterol to rabbits, showed that an inflammatory reaction consisting of edema and cellular infiltration preceded the formation of concrements. Sjodahl et al. (70) further showed that

lysolecithin (the product of enzymatic cleavage of lecithin) induced an inflammatory reaction when instilled into the lumen of rabbit gallbladders. Against this hypothesis, is the absence of these changes in some human gallbladders surgically removed for cholelithiasis (64). Furthermore, Borgstrom et al. (71), using the rabbit model, found none of the inflammatory changes reported (69) and hence concluded that inflammation could not account for gallstone formation.

Inflammatory and mucosal changes have long been regarded as a result rather than the cause of gallstones, probably because most specimens obtained during cholecystectomy for gallstones represent advanced stages of the disease. Also, definition is still confounded by failure to reconcile the criteria for cholecystitis among pathologists (65) and in animal models (72). One current view is that some form of gallbladder epithelial injury takes place before gallstone formation but is not manifested by the classical histologic signs of acute inflammation (e.g., cellular infiltration); the damaged epithelium may increase mucus secretion and cellular proliferation, as in other gastrointestinal mucous membranes (64).

The role of mucus in the pathogenesis of cholesterol gallstones was first stressed by Womack and his colleagues (48). They demonstrated mucopolysaccharides in the center of human gallstones, and in histochemical studies showed a thin layer of mucoid substance covering the gallbladder epithelium. They concluded that a prosthesis of mucus was necessary for stone formation. Maki et al. (73) confirmed these findings and identified the mucous substances as sulfated glycoproteins, and Bouchier et al. (74) reported that the mucus content was greater in bile from calculous gallbladders than from normal ones. Lee et al. (75)

reported similar findings and concluded that these macromolecules were secreted into bile by the gallbladder mucosa. Further interest in the role of mucus in the evolution of gallstones stemmed from observations in animal studies. Hayward et al. (76) who fed dihydrocholesterol to rabbits, demonstrated that during lithogenesis the normally nonsecretory epithelium became secretory, producing excess mucus before the formation of stones. Similar findings have been documented in other animal species: for instance in hamsters fed a fat-free diet (77), mice fed cholesterol cholic acid (64, 78, 79), prairie dogs fed a cholesterol diet (50, 80), and in mongrel dogs during dietary induction of pigment gallstones (81). The unique result was increased secretion of mucus preceding the formation of stones, despite wide variation in the method of induction and the composition of the stones.

Mucus appears to play a crucial role in gallstone formation: 1. It functions as a nucleating agent (48, 50); 2. It increases bile viscosity, trapping microcrystals and preventing their evacuation (7, 63, 82); 3. It provides scaffolding for the further deposition of crystals (81, 82); and 4. It probably sequesters bile salts, reducing the quantity available for micelle formation (82). This hypothesis was supported by the fact that no gallstones were formed after mucus secretion had been blocked by aspirin in cholesterol-fed prairie dogs (83). In recent experiments, however,lactulose prevented gallstone formation in hamsters without any obvious change in the concentration of gallbladder mucus (84).

The nature of the stimulus that initiates the excessive mucus production, and the mechanism by which it releases mucus into the gallbladder lumen, are still not clear. In two studies with different

animal models (50, 82), exclusion of the gallbladder from the enterohepatic circulation by cystic-duct ligation did not result in mucus hypersecretion. This suggests that the factor which stimulates mucus synthesis probably circulates in the bile. In other experiments, in dihydrocholesterol-fed rabbits, Lee and Scott (72) found mucosal changes (i.e., mucus hypersecretion) coincident with an increased proportion of allodeoxycholate; this bile acid precipitated to form stones, indicating that it was the initiating factor. Bile acids are injurious to most epithelial surfaces, a property dependant upon their composition and concentration (85), and there seems no reason why they should affect gallbladder epithelium differently. Wahlin and his colleagues (86) showed that the synthesis and secretion of mucus by the principal cells of mouse gallbladder epithelium is influenced by cholecystokinin as well as by cholinergic drugs (87). However, the absence of mucus hypersecretion after ligation of the cystic duct, clearly excludes these agents as initiators of this process.

Another mucosal change observed in the gallbladder during lithogenesis is increased cellular proliferation. Autoradiography in vitro after ^{3}H-thymidine labeling showed an increase in both DNA synthesis and the mitotic index in the epithelium of gallbladder from patients who had cholelithiasis (88). Studies of mice fed a lithogenic diet (cholesterol cholic acid) revealed cellular proliferation as early as 48 hours (64) - a finding confirmed with the same model by Ziegler and Palme (89) who also noted its association with excessive exfoliation of epithelial cells into the gallbladder lumen when no concrement had formed. There have been reports of cellular hyperplasia in the bile duct during the induction of choledocholithiasis in rats fed lithocholic

acid (90) and in the gallbladder during stone formation in several other species (91).

As for mucus hypersecretion, the initiating stimulus for increased kinetic activity of the epithelium is not known. Many theories have been proposed. One of these maintains that distension of the gallbladder may be responsible for increased cell renewal (92), a hypothesis supported by the increased mitotic activity in the distended gallbladder of patients in whom the distal end of the common bile duct is obstructed by neoplasm (93). Furthermore, in mice fed a lithogenic diet, the gallbladder starts to enlarge at 2 days (64) indicating that the distension may lead to transient ischemia (92) and cell damage, the release of lysosomal enzymes, and thus an inflammatory reaction (94). However Ziegler and Palme (89) could find no histologic evidence of ischemic reaction with cellular damage; in fact, they reported that increased cell renewal preceded distension of the gallbladder, precluding the latter as the initiating agent.

Another school of thought proposes changes in bile acid composition as the factor responsible. This theory is backed up by the observation that in mice the ingestion of cholic acid or deoxycholic acid, but not of cholesterol, increases the uptake of ^{3}H-thymidine in gallbladder epithelium, and fibroblasts in the lamina propria and hepatocytes (89). However, it is not certain whether bile acids affect the cell renewal process directly or whether the increased uptake represented a response to epithelial cell injury. Recently, it has been shown that cerulein (a cholecystokinin analog) and cholecystokinin-octapeptide have the same effect as these bile acids on mouse gallbladder epithelium (95). Polypeptide hormones and hormone-like mediators are known to

initiate, promote, and maintain proliferative responses in certain disease states (96); however, no specific role has been ascribed to cholecystokinin in experimental cholelithiasis.

Alterations in fluid transport across the gallbladder wall constitute another mucosal aberration that can enhance the precipitation of gallstones during lithogenesis. Fluid transport was increased in gallbladders of rabbits and guinea pigs during induction of cholelithiasis (97). This occured concurrently with increased cellular proliferation and mucus hypersecretion and preceded any histologic evidence of inflammation. In these circumstances it is likely that cholesterol precipitation is facilitated by the hydrostatic pressure, which would tend to keep any microcrystal adherent to the epithelium - the site where stone formation is known to begin (97). In studies of mice, Tepperman and Weiner (94), reported faster hepatic production of bile and increased cholesterol concentration in gallbladder bile, which they attributed to altered fluid transport across the gallbladder wall. Others have shown that gallbladders with histologic features of inflammation absorb bile salts, rendering the gallbladder bile more lithogenic (98, 99). In specimens with advanced pathologic changes, however, absorptive function of the epithelium is impaired (100, 101).

CONCLUSION

Gallstones form in stages, each necessary for the next (102). Many factors operate at the different stages to bring about changes that enhance stone formation. In the first stage, the liver produces lithogenic bile whose basic biochemical defect is decreased bile-salt concentration relative to cholesterol. Interruption of the

enterohepatic circulation, also facilitates lithogenicity of bile, by decreasing hepatic production of bile salts relative to cholesterol. The second stage takes place in the gallbladder, which provides optimal conditions for nucleation, the precipitation of excess cholesterol into microcrystals, and growth of the crystals to macroscopic stones. In this regard, mucous substances have been strongly implicated as specific nucleating agents. Aberration of fluid transport that follows epithelial changes, also, facilitates this process. Decreased gallbladder motility leads to stasis, enhancing the retention of precipitates and permitting their growth into macroscopic stones. The latter may remain 'silent', or produce symptoms by inducing cholecystitis or obstructing the cystic duct.

THE PRESENT STUDY

Overall Objective

The main objective of this research was to study early morphological and histochemical changes in the gallbladder epithelium in response to a lithogenic stimulus (dietary cholesterol).

Background Knowledge

Recent studies of the pathogenesis of cholesterol gallstones have shown that the production of lithogenic bile (i.e., supersaturated with cholesterol) is necessary to stone formation (45) and that the gallbladder, also plays a primary role by providing optimal conditions for the nucleation and precipitation of cholesterol microcrystals and their growth to macroscopic stones (49, 50). However, our knowledge on this subject is still far from complete. This is partly because prospective studies designed to define the stages of evolution of the disease in humans are technically difficult and are complicated by ethical considerations.

The only large scale prospective studies have been conducted among the Pima Indians, a tribe with a very high prevalence of gallstones. Bile saturation increases significantly during pubertal growth and development and by the age of 19 years, 71% of females and 29% of males produce lithogenic bile but do not form stones (103). About 8 years later, well over half of them have gallstones detectable on cholecystography (5). Thus there is clearly a latent period between the

onset of production of lithogenic bile and the formation of stones. The interim changes that bring about the nucleation and precipitation of excess cholesterol from the lithogenic bile remain speculative but probably they occur in the gallbladder, the commonest site of cholesterol gallstone formation (63, 89). Knowledge of these events would aid delineation of the pathogenesis of the disease and thus the development of preventive and therapeutic measures.

Retrospective studies in humans have not resolved this problem, because they cannot discriminate between changes relating to 'initiation' of stones and those of the subsequent 'growth' phase (81). Moreover, findings on specimens removed at operation for cholelithiasis reflect mainly end-stage processes of the disease and reveal nothing about the early stages. This situation has necessitated the use of animal models to formulate concepts of the disease mechanism in humans.

Recent results of induction of gallstones in various animal species have clearly implicated changes in gallbladder epithelium (e.g., mucus hypersecretion) during the latent period as nucleating agents (50,64). However, the type of mucus produced is not known. With the exception of the recent report by Ziegler et al. (79) of acute studies (15-30 hr) in mice fed a diet rich in cholesterol and cholic acid, information on this subject is based on findings some days or weeks after the start of the lithogenic diet. Thus there is scanty information on the very early mucosal changes in the gallbladder during lithogenesis, information that would aid in the elucidation of underlying mechanisms.

The present research investigated these early changes in the structure of gallbladder epithelium in response to a lithogenic

challenge [dihydrocholesterol (DHC)] in rabbits.

SPECIFIC AIMS

1. To determine gross structural changes in size (using volume displacement) and changes in the appearance of the luminal surface of the gallbladder; and to note changes in the color of bile and the development of crystals.

2. To determine morphological changes in the mucosa, using light microscopy and scanning and transmission electron microscopy.

3. To determine histochemical changes during this early phase, using PAS and Alcian Blue (pH 2.5) stains.

4. In correlating the findings in aims 1-3, to outline the sequence of early changes in the gallbladder epithelium at both the cellular and the subcellular level in response to the lithogenic stimulus.

SELECTION OF THE ANIMAL MODEL

Rabbits tolerate the dihydrocholesterol-rich diet very well (104) and their gallbladders provide sufficient tissue for the investigations performed in this study. Furthermore, the dihydrocholesterol-rabbit model is especially useful for short-term studies as stones are produced very quickly in most animals (104) so that morphological alterations are likely to occur very early during lithogenesis.

Extrapolation of the findings in an experiment of this type to human gallstone disease is limited by the following:

1. Human gallstones are mainly mixed stones, predominantly cholesterol (10), whereas in rabbit fed DHC the major constituent is allodeoxycholic acid, an insoluble isomer of the normal deoxycholic acid (104).
2. The composition of the lithogenic diet is not identical to that consumed by humans.
3. Induction of gallstones in this model is acute, whereas the formation of gallstones in humans is almost certainly a gradual process over a long time - a fact supported by the studies in Pima Indians (5,103).

Nevertheless, it was hoped that the findings in these experiments would stimulate the development of concepts of the pathogenesis of gallstone disease in humans.

MATERIALS AND METHODS

A total of 36 female, white New Zealand rabbits weighing 1.4 to 2.5 kg were used for this study; 30 were experimental and 6 were controls. The animals were housed in separate cages and were allowed water *ad libitum*. Experimental animals were fed a daily diet of 100g of rabbit chow pellets (Purina) containing 1% w/v of dihydrocholesterol (DHC) (5-cholestan-3β-ol, Aldrich Chemical Company, Milwaukee, U.S.A.) which had been dissolved in ethyl ether. The diet was mixed while damp and allowed to dry at room temperature in a hood. Control animals were fed the same amount of pellet to which only ethyl ether was added and allowed to dry as above. The animals were fed at 8:30 a.m. daily except those scheduled for sacrifice.

The gallbladder was removed from 6 experimental animals after 1, 2, 3, 4 and 5 days on the DHC-rich diet and from the controls on the fifth day. Anesthesia was induced with halothane and the biliary tract was exposed through a midline incision. The cystic duct was double-clamped, divided and the organ was gently removed by blunt dissection; the rabbits were killed immediately by cardiac puncture. The gallbladder was placed in a finely calibrated cylinder containing freshly prepared Kreb's solution and the clamp removed. The volume of solution displaced was recorded as the size of the gallbladder and its contents. The organ was opened longitudinally, thoroughly washed in Kreb's solution, gently pinned to a piece of cork without stretching and cut into half.

One half of each gallbladder was used for light microscopy and histochemistry. The tissue was fixed in formalin for 24 hours and then

embedded in paraffin. Sections were cut at 8μm and stained with hematoxylin and eosin and examined with a Leitz Orthoplan photomicroscope. Periodic Acid-Schiff (PAS) and Alcian blue (pH 2.5) histochemical reactions were performed on other paraffin-embedded tissue to demonstrate neutral and/or acidic mucopolysaccharide material' respectively.

The other half of each gallbladder was processed for transmission (TEM) and scanning (SEM) electron microscopy. It was floated face-down in Karnovsky's cacodylate-buffered (gluteraldehyde-paraformaldehyde) fixative at pH 7.4 for 24 hours. Specimens for TEM were cut from the edges of these blocks and diced into appropriately sized pieces and the remaining tissue block was processed for SEM. Both sets of tissue were postfixed in 2% cacodylate-buffered osmium tetroxide and dehydrated through an alcohol series to absolute ethanol. TEM samples were passed through absolute acetone and embedded in Spurr's resin. Thin sections were cut on a Reichert-Jung ultracut ultramicrotome, mounted on 300-mesh copper grids, counter-stained with uranyl acetate and lead citrate and examined in a Philips 410 TEM at 80Kv. Thick sections (1μm) were obtained with the same ultramicrotome, stained with toluidine blue and examined with a Leitz Orthoplan photomicroscope. From absolute ethanol, tissues from SEM were critical point dried in a Seevac carbon dioxide critical point dryer. The samples were then mounted on aluminum stubs, sputter coated with gold and examined using a Philips 505 SEM at 20Kv.

RESULTS

All rabbits tolerated the diet well and appeared healthy throughout the experimental period.

There was no correlation between the size of the gallbladder and the number of days the animal had been on the DHC-rich diet (Table 1). In gallbladders from control animals, the bile was clear green and contained no crystals and the mucosa had a uniform, velvety-like appearance. No mucosal lesions were seen macroscopically in any of the specimens from animals fed DHC-rich diet. The gallbladder bile from experimental animals was clear green and contained no crystals.

Table 2 summarizes the morphological change of gallbladder epithelium from animals fed the DHC-rich diet.

LIGHT MICROSCOPY

a) Paraffin Sections

The mucosa of gallbladders from control rabbits appeared normal. Mucosal folds of varied height and width were lined by tall columnar epithelial cells with basal nuclei. The basal lamina separated the epithelium from the lamina propria, which consisted of loose connective tissue. No glands were observed. In tissues from the experimental group, the most remarkable finding was oval intra-epithelial vesicles (Fig. 2). The vesicles were observed as early as day 1 and their number increased in subsequent specimens. In some parts of the epithelium they had ruptured and were in direct communication with the gallbladder lumen. Higher magnifications revealed that in some epithelial cells

TABLE I: CHANGE IN SIZE OF GALLBLADDER IN RELATION TO DURATION OF DIET

Group	Days on Diet	Mean Volume Displaced* (ml)
Experimental	1	0.83 ± 0.06
	2	1.42 ± 0.21
	3	0.90 ± 0.19
	4	1.02 ± 0.07
	5	0.88 ± 0.04
Control	day 5	1.15 ± 0.12

* Mean ± SEM: n = 6 animals per sampling interval.

TABLE 2: SUMMARY OF MORPHOLOGICAL CHANGES IN GALLBLADDER EPITHELIUM OF RABBITS FED DIHYDROCHOLESTEROL-RICH DIET

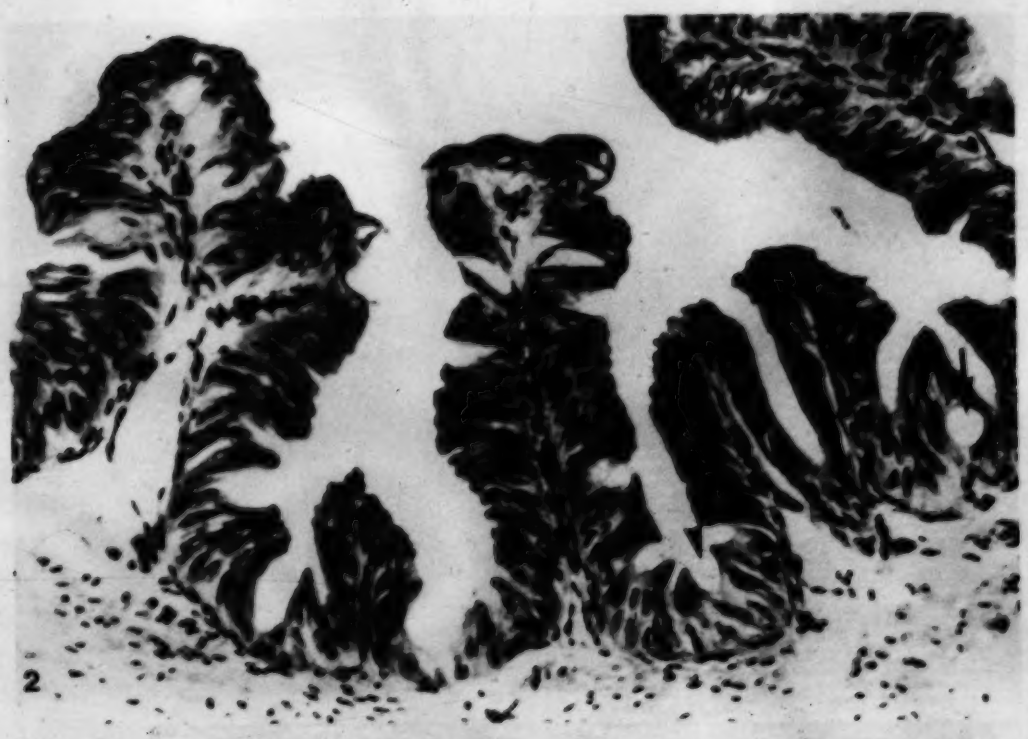
	Observations	DURATION ON DIET Day 1	Day 2	Day 3	Day 4	Day 5	Control (Day 5)
LM	-intraepithelial vesicles	+++	+++	+++	+++	+++	+
	-osmiophilic lipid droplets	++++	++	+	-	-	-
	-pale staining cells	++	+++	+++	+++	++++	+
	-edema of lamina propria	+	++	++	+++	+++	-
HISTOCHEM.	-A/Blue stain	+++	+++	+++	+++	+++	+
	-PAS stain	++	++	++	++	++	+
SEM	-Focal areas of epithelial exfoliation; degenerating cells with short, stubby microvilli	++	+++	++++	+++	++++	+
TEM	-Subapical granules and dilated smooth ER.	+++	+++	++++	++++	++++	+
	-Pale-staining cells with widely dispersed organelles	++	+++	+++	+++	++++	+
	-Destruction of microvilli with blebbing of apical plasma membrane	+	++	+++	+++	++++	+
	-Dilated lateral intercellular space; edema of lamina propria	++	++	++	+++	++	+
	-Focal areas of discontinuity in epithelial sheet with cellular debris in G.B. lumen	++	++	+++	+++	++++	+

FIGURE 2: Light micrograph of gallbladder epithelium 24 hours after ingestion of DHC-rich diet, showing a large intraepithelial vesicle (arrowed). Some have ruptured and their lumina are continous with that of the gallbladder (arrowhead).

H & E x400

FIGURE 3: Light micrograph of gallbladder epithelium at day 3. The lamina propria is edematous and a blood vessel is engorged with erythrocytes. Vasculitis and perivascular infiltration are absent.

H & E x400



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the cytoplasm was clear and contained only the nuclei. No Rokitansky-Aschoff sinuses were seen in any of the gallbladders. The lamina propria and fibromuscular layer were edematous (Fig. 3). Most of the blood vessels in the lamina propria were dilated and some were engorged with erythrocytes, but the endothelial cells appeared normal. There were no hemorrhages or perivascular infiltration associated with these vessels.

Histochemical studies with PAS and Alcian blue (pH 2.5) stains revealed mucus covering the epithelial surface and filling the intra-epithelial vesicles (Fig.4a,b) H & E stained the mucus poorly, but PAS and Alcian blue stained intensely. This finding, indicating both neutral and acidic mucopolysaccharide, was more marked in experimental than control animals (Fig 5a,b).

b) Thick Plastic Sections

In preparations from control animals, some parts of the mucosal fold contained lateral intercellular spaces.

In gallbladders from rabbits fed DHC, at day 1 (and rarely thereafter) the most striking feature was the deposition of large osmiophilic droplets between the epithelial cells and the basement membrane and within underlying foam cells in the lamina propria (Fig. 6a). In the latter, they had pushed the cells' nuclei to the periphery of the cytoplasm (Fig. 6b,c). The epithelial cells contained tiny cytoplasmic vacuoles mostly situated supranuclearly, but otherwise they appeared normal in most cases. The extracellular lipoid material released into the gallbladder lumen through foci of discontinuity in the epithelium (Fig. 7). In gallbladders examined at day 2 and later, cellular damage was more intense and the lamina propria appeared

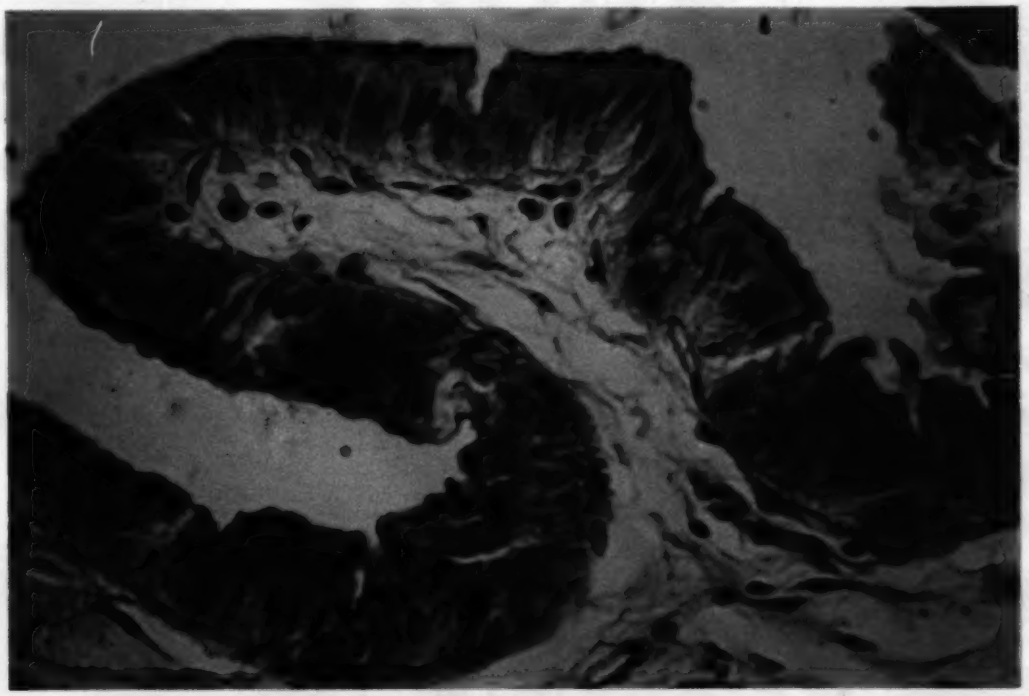
FIGURE 4: Gallbladder epithelium from rabbit fed the DHC-rich diet for 24 hours showing:

Figure 4a. Acidic (blue-staining) mucopolysaccharides lining the surface as well as the intraepithelial vesicle.

Alcian Blue x400

Figure 4b. PAS staining demonstrating neutral (purple-staining) mucopolysaccharides inside the intraepithelial vesicle.

PAS x400



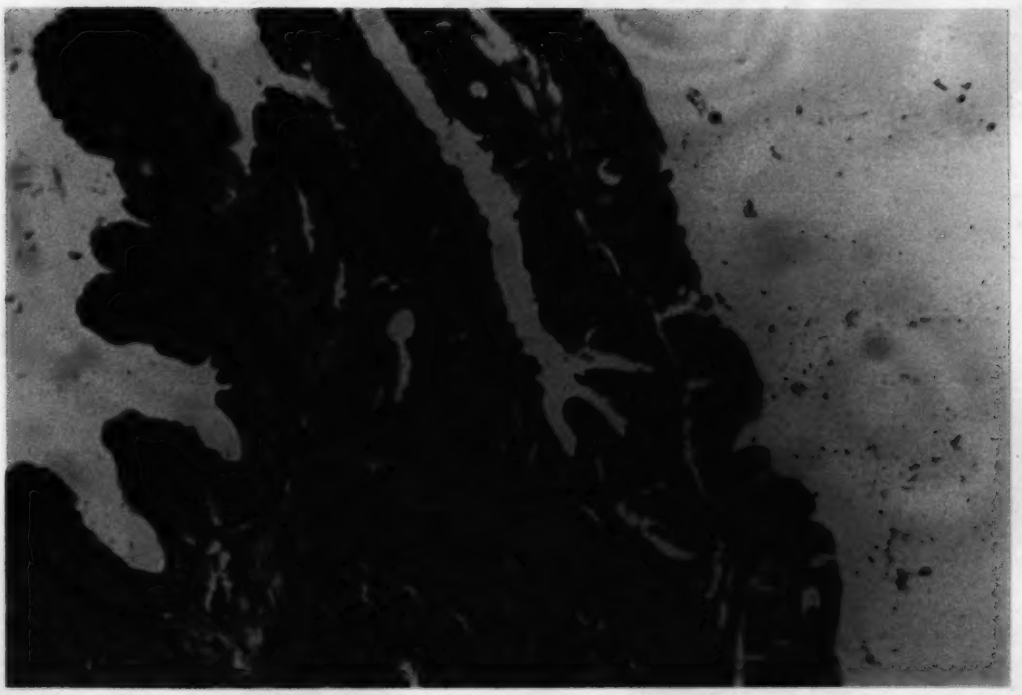


FIGURE 5: Gallbladder epithelium from a control rabbit demonstrating the paucity or abscence of mucopolysaccharides on both:

Figure 5a. Alcian Blue x400

Figure 5b. PAS stains. x400

FIGURE 6: Thick plastic sections of gallbladder epithelium demonstrating lipid vacuoles 24 hours after the rabbit had ingested DHC-rich diet.

Figure 6a. The droplets (arrowhead) are present between the plasmalema and the basal lamina (arrow) as well as in foam cells in the lamina propria.

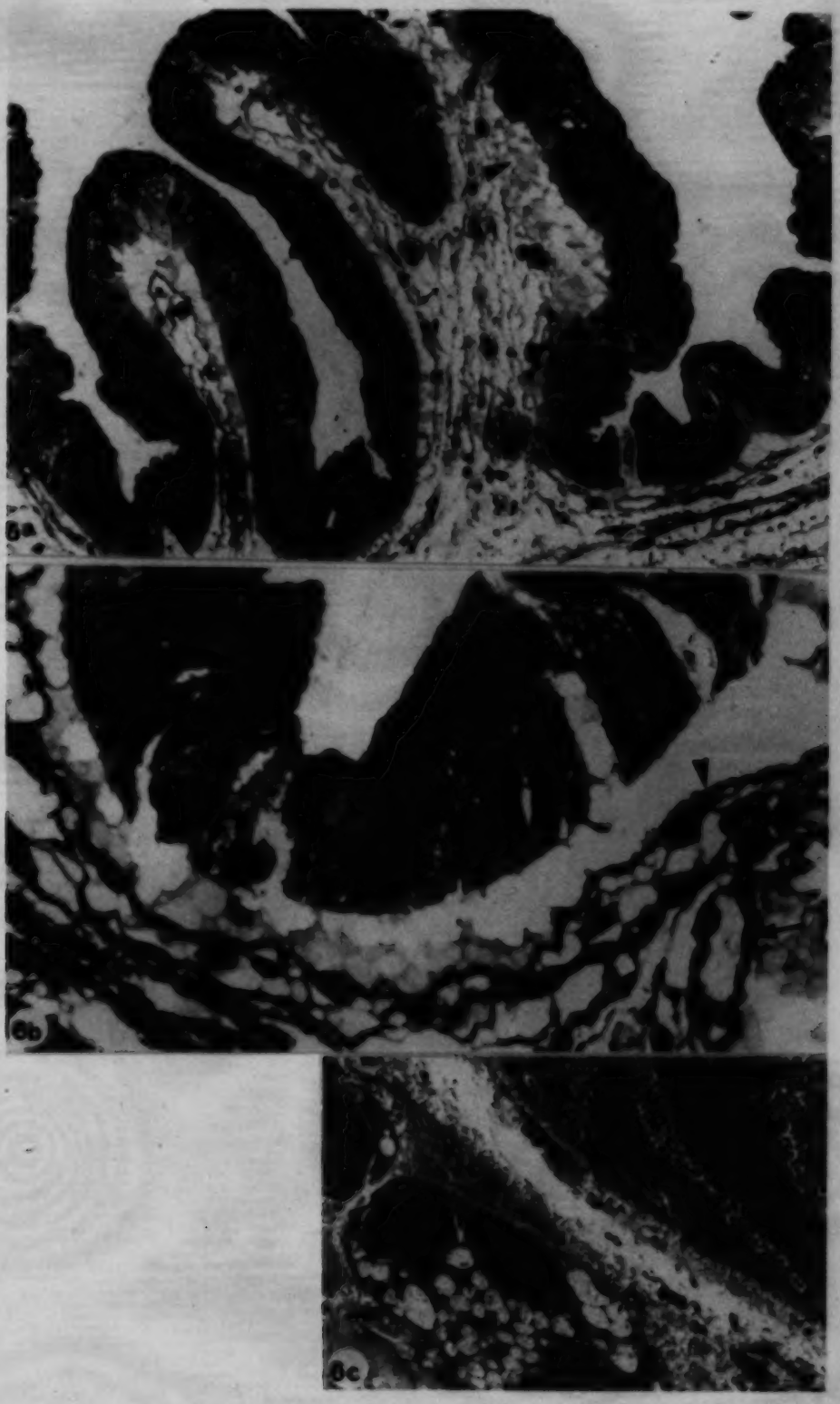
Toluidine blue x400

Figure 6b. The basal lamina (arrowhead) is markedly displaced into the lamina propria and the nuclei of the foam cells are pushed to the periphery of the cytoplasm (arrow).

Toluidine blue x400

Figure 6c. Transmission electron micrograph of gallbladder epithelium showing the accummulation of the lipid droplet in the cytoplams of a foam cell (arrow)

x1650 (9,900)


8a
8b
8c

edematous. Cells that had undergone intensive internal disruption stained poorly and were observed predominantly in the upper third of the mucosal fold (Fig. 8, 9).

ELECTRON MICROSCOPY

a) Scanning Electron Microscope (SEM):

In preparations from control gallbladders, low power Scanning electron micrographs showed marked folding of the mucosa with surface creases (Fig. 10a). At higher magnification, individual cellular outlines were prominent, imparting a cobblestone appearance to the surface (Fig. 10b). The apical surface of the cells appeared slightly convex and was covered with moderately developed microvilli (Fig. 10c).

In specimens from experimental rabbits, at days 1 and 3 (Fig. 11a,b) the surface was broken by many gaps left by the extrusion of epithelial cells. The gaps were more numerous on the top of the mucosal folds than in the valleys - a feature that concurred with findings on thick-plastic sections. Intercellular spaces around degenerating cells were widened (Fig. 11b) and at higher magnifications, the microvilli appeared short and stubby (Fig 12a). Some cells were partly or completely devoid of microvilli and their apical plasma membrane were exposed. (Fig. 12b). In tissues examined at day 5, there were large areas of epithelial desquamation and associated cellular debris (Fig. 13a). The basement membrane exposed by this desquamation was continuous, but appeared indented by marks left by the extruded cells (Fig. 13b).

b) Transmission Electron Microscopy (TEM):

In preparations from control gallbladders, the TEM revealed two

FIGURE 7: Thick-plastic sections of the gallbladder epithelium demonstrating the escape of the lipid vacuoles (arrow) into the gallbladder lumen through a point of discontinuity at the top of a mucosal fold.

Toluidene blue x400.

FIGURE 8: Thick-plastic sections of the gallbladder epithelium 2 days after the rabbit had ingested the DHC-rich diet. Degenerating cells stained palely and occurred predominantly on the upper third of the mucosal fold; the lamina propria is edematous.

Toluidene blue x400

7

8

FIGURE 9: Thick-plastic sections of gallbladder epithelium 3 days after ingestion of the DHC-rich diet showing edema of the lamina propria, and pale staining degenerating cells.

Toluidene blue x400

FIGURE 10a: Scanning electron micrograph of the gallbladder epithelium of a control rabbit demonstrating marked folding of the mucosa and the surface creases.

x194

9

10a

FIGURE 10: Scanning electron micrograph of gallbladder epithelium from a control rabbit showing:

Figure 10b. The prominence of cellular outlines.

x356

Figure 10c. Microvilli covering the apical surface of the individual cells.

x1690

10 b

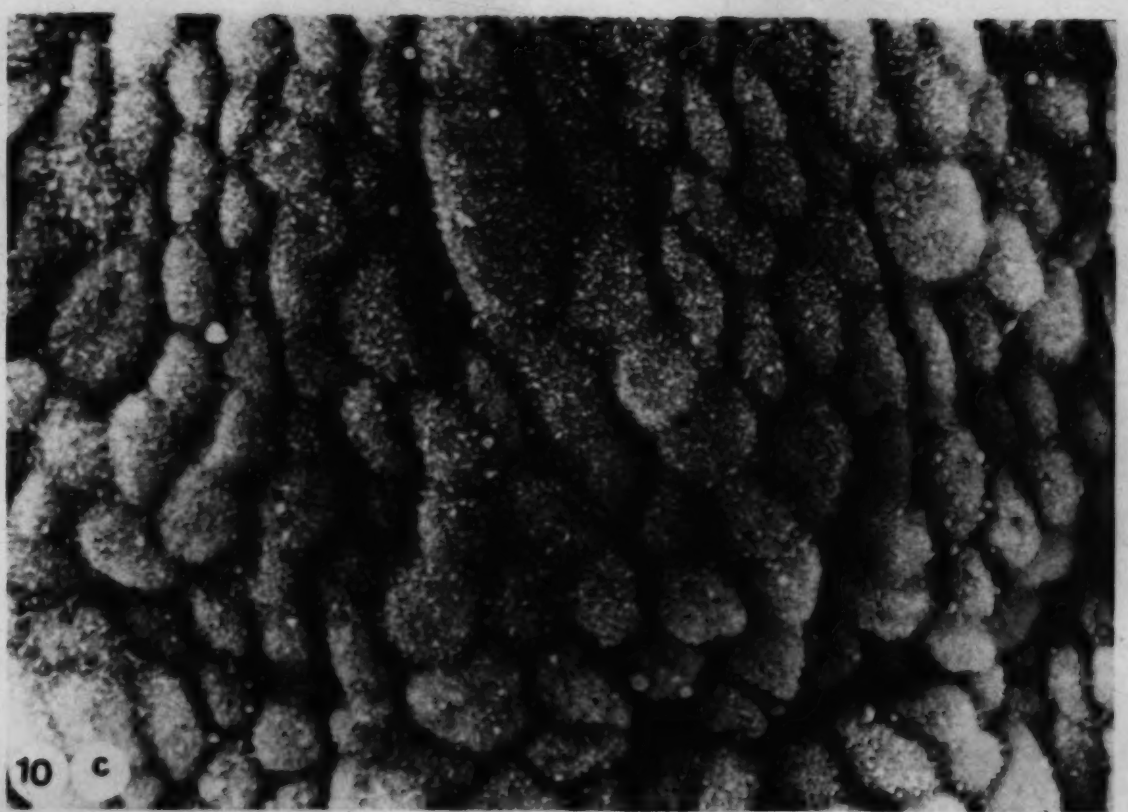

10 c

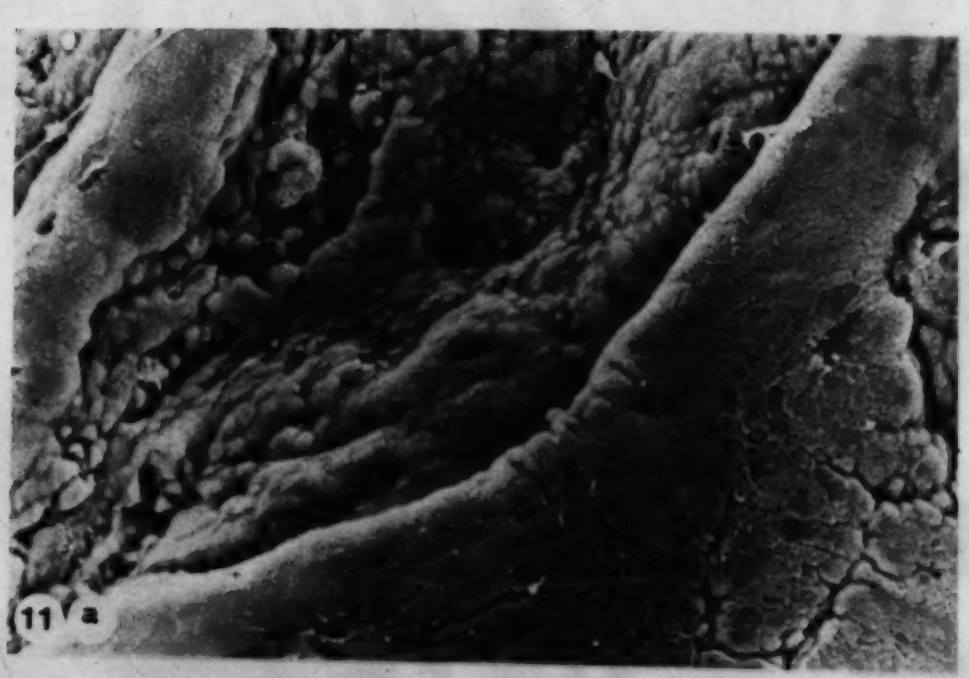
FIGURE 11: Scanning electron micrograph of the gallbladder epithelium of rabbits fed the lithogenic diet for:

Figure 11a. Day 1, showing focal areas of cellular damage(arrow).

x424

Figure 11b. Day 3, demonstrating gaps left by extruded cells and widened apical intercellular space around some cells (arrow).

x710


11 a

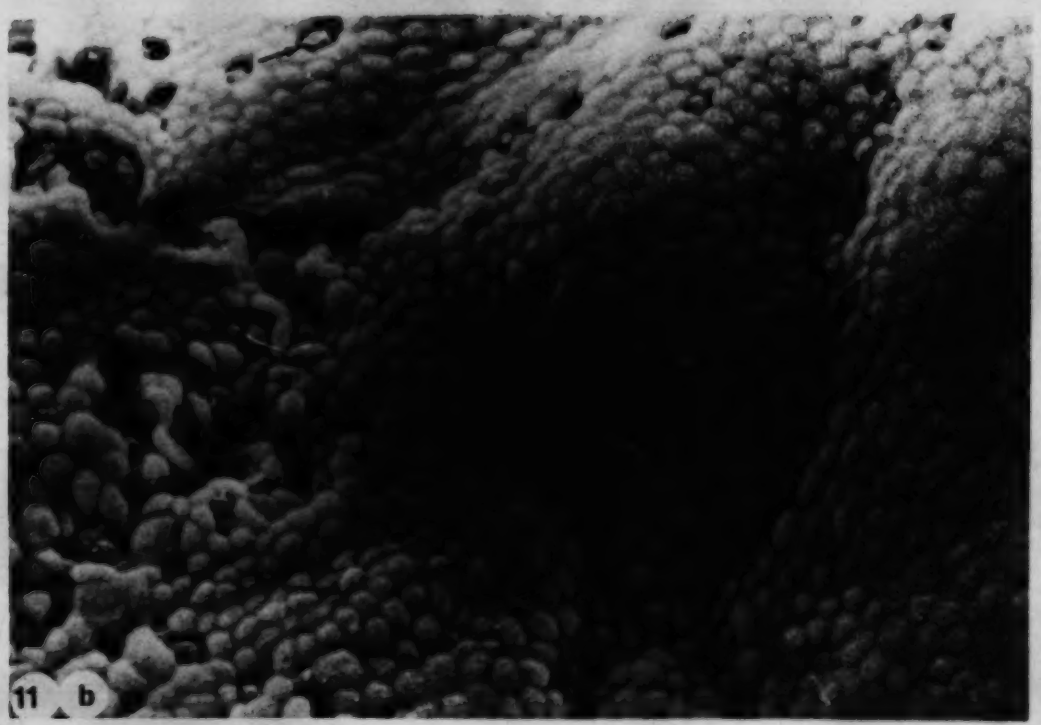

11 b

FIGURE 12: High-power scanning electron micrograph of the gallbladder epithelium 4 days after ingestion of the DHC-rich diet demonstrating:

Figure 12a. An extruding degenerated cell (arrow) with short stubby microvilli.

x3280

Figure 12b. Paucity or absence of microvilli (arrow) from the apical surfaces of some cells.

x3500

12 a

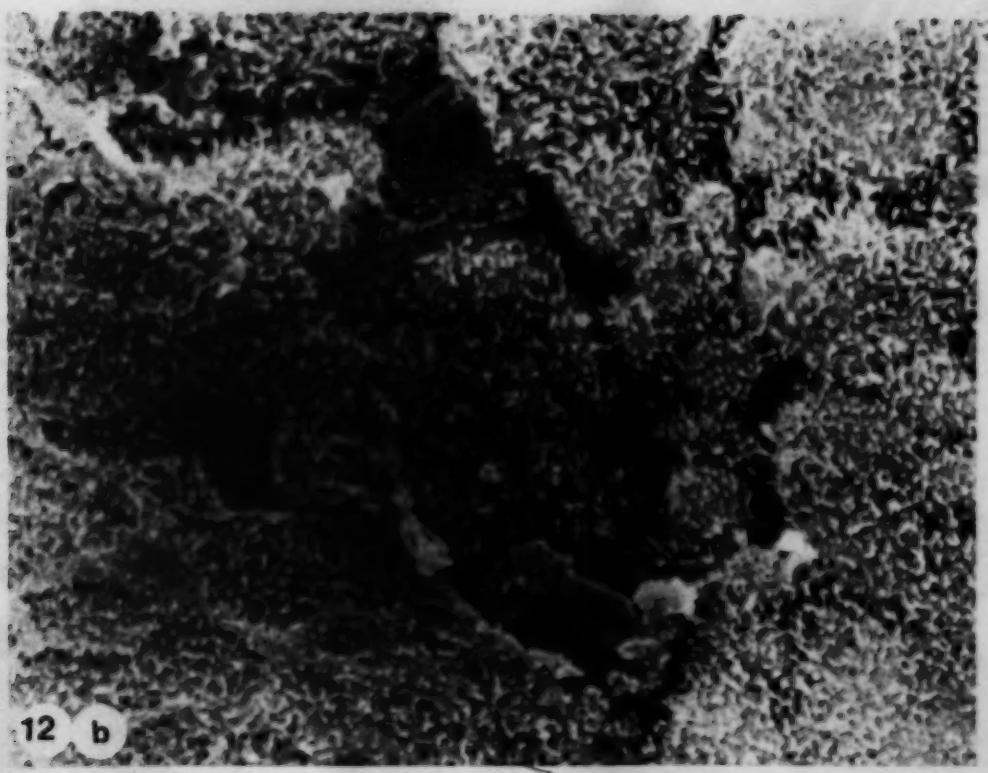

12 b

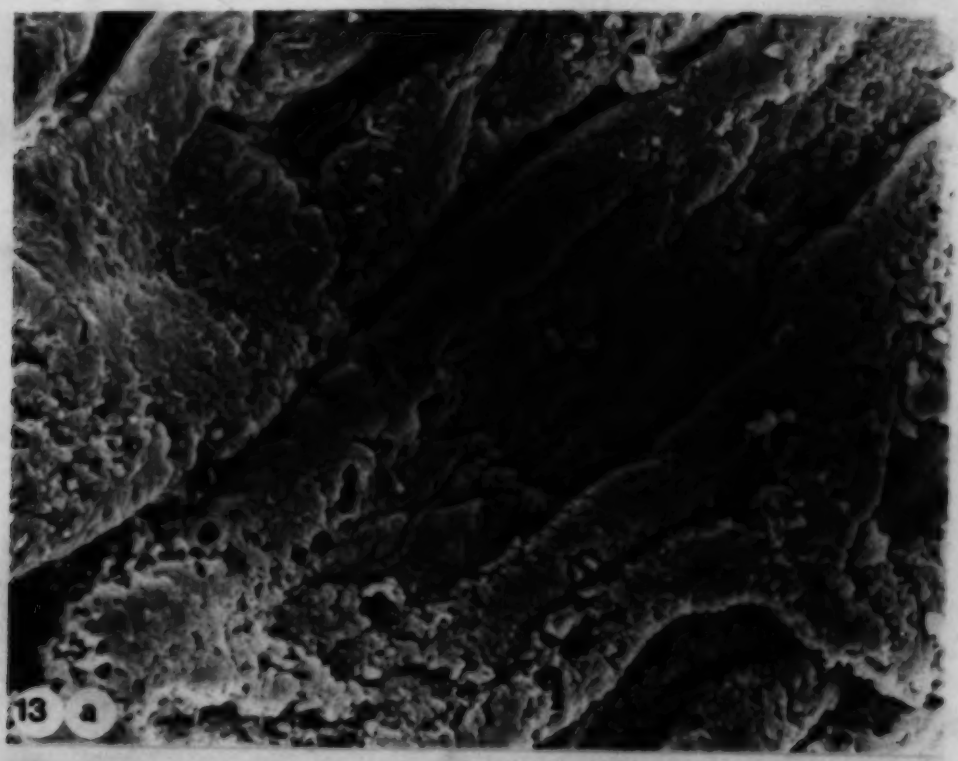
FIGURE 13: Scanning electron micrograph of gallbladder epithelium at day 5 show:

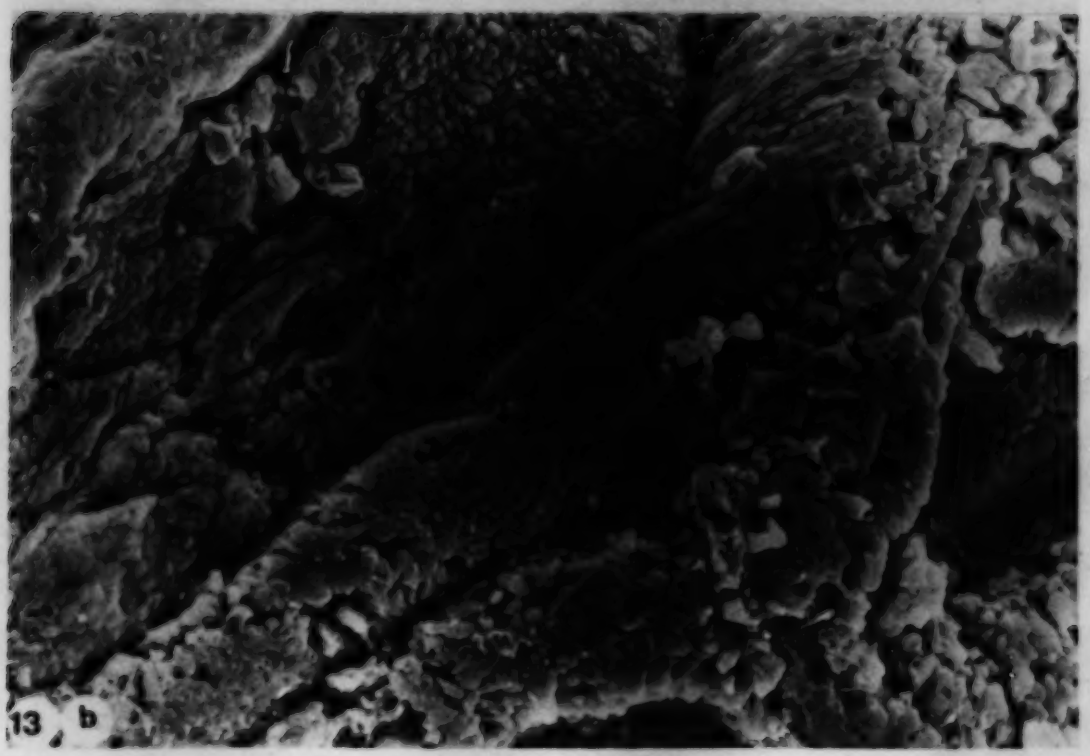
Figure 13a. Large areas of epithelial cell damage.

x260

Figure 13b. The surface of the basal lamina (arrow) is denuded of epithelial cells.

x280


13 a


13 b

types of epithelial cells in the mucosa. The most predominant type was the tall columnar cells, with slightly electron-lucent cytoplasm. The apical plasma membrane appeared slightly convex and was covered by moderately developed microvilli (Fig. 14a). Immediately beneath the luminal plasma membrane was the organelle-free apical zone; mitochondria were widely distributed in the cytoplasm except in the apical zone. The second epithelial cell type was the 'pencil cell', which was characterized by its narrowness and electron-dense cytoplasm (Fig 14b). Like the columnar cell, it extended from the basal lamina to lumen and also bore microvilli at its apex.

In preparations from gallbladders of the experimental animals, cellular alterations were mainly cytoplasmic or intracellular and consisted of many mucous glycoprotein vesicles and dilated smooth endoplasmic reticulum (Fig. 15a). The vesicles, mostly supranuclear, developed in the Golgi region at the apical pole and to the side of the nucleus. Most of these had accumulated in the lower margin of the apical zone, reducing its width; others had crossed the zone and fused with the apical plasma membrane (Fig. 15b). Cells that had undergone extensive internal disruption appeared edematous, with highly electron-lucent cytoplasm and widely dispersed organelles (Fig. 16). Despite their severe internal degeneration, some of these cells remained attached by their foot process to the basement membrane. Ultrastructural changes in microvilli, plasma membrane, and tight junctions of the epithelial cells occurred predominantly in those cells whose mitochondria were swollen. These cells had fewer, stubby microvilli, and their apical plasma membrane was blebbed (Fig. 17a,b) - features similar to those associated with degenerating cells on SEM.

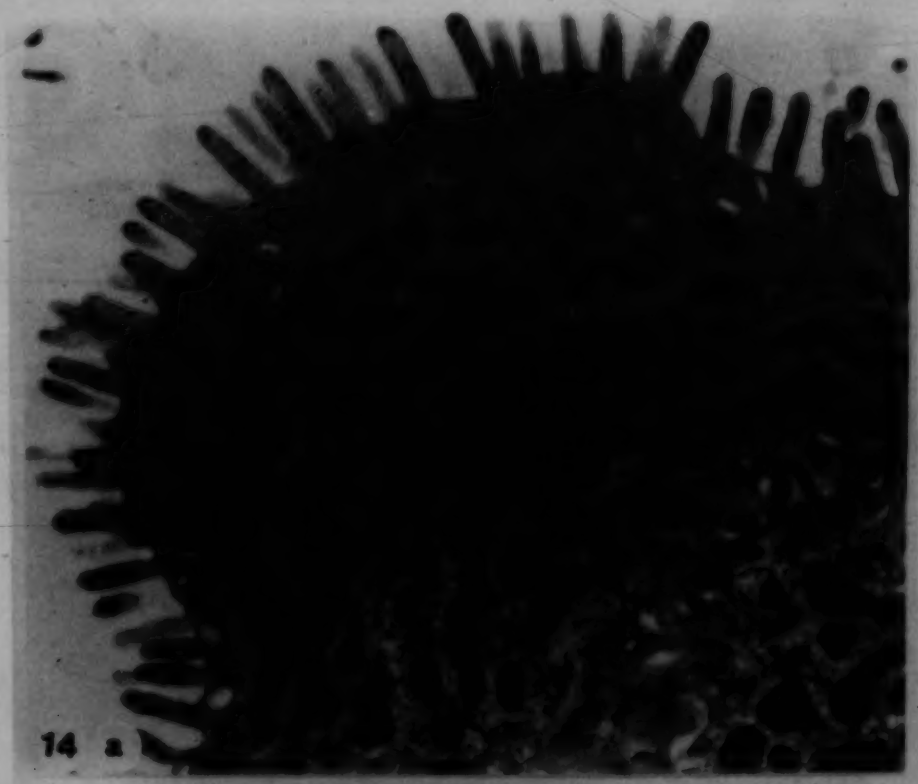
FIGURE 14: Transmission electron micrograph of gallbladder epithelium from a control rabbit showing:

Figure 14a. The apical part of an epithelial cell containing an organelle-free zone and shows the typical distribution and appearance of the mitochondria.

x2500

Figure 14b. The basal part of epithelial cells showing a 'pencil' cell with dark-staining cytoplasm and columnar cells with electron-lucent cytoplasm.

x2300


14 a

14 b

FIGURE 15: Transmission electron micrograph of gallbladder epithelium 24 hours after ingestion of the lithogenic diet.

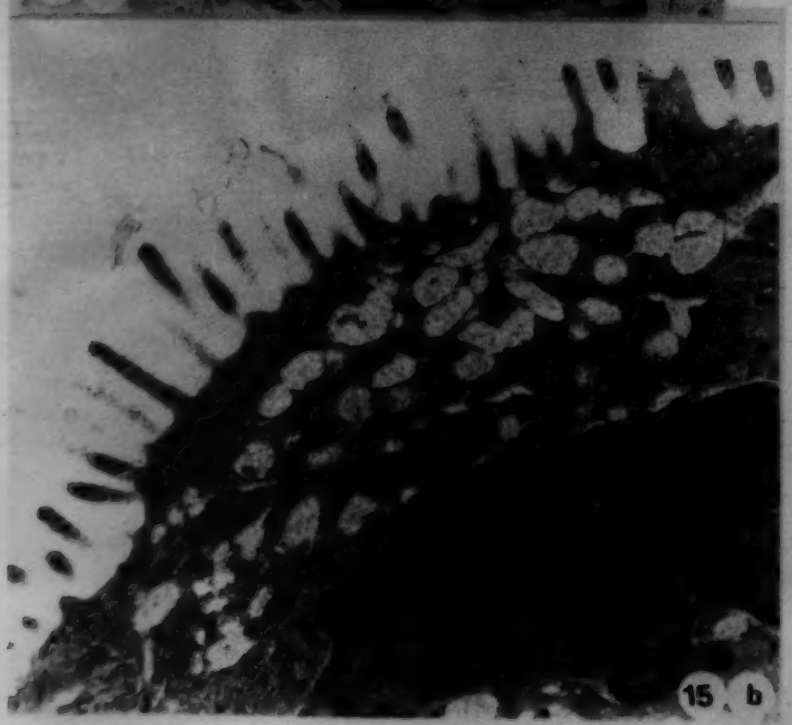
Figure 15a. Mucus vacuoles (big arrow) are present and the endoplasmic reticulum is dilated (small arrow).

x2300

Figure 15b. Mucus vacuoles have accummulated in the apical zone of the cytoplasm, reducing its width.

x3500

15 a



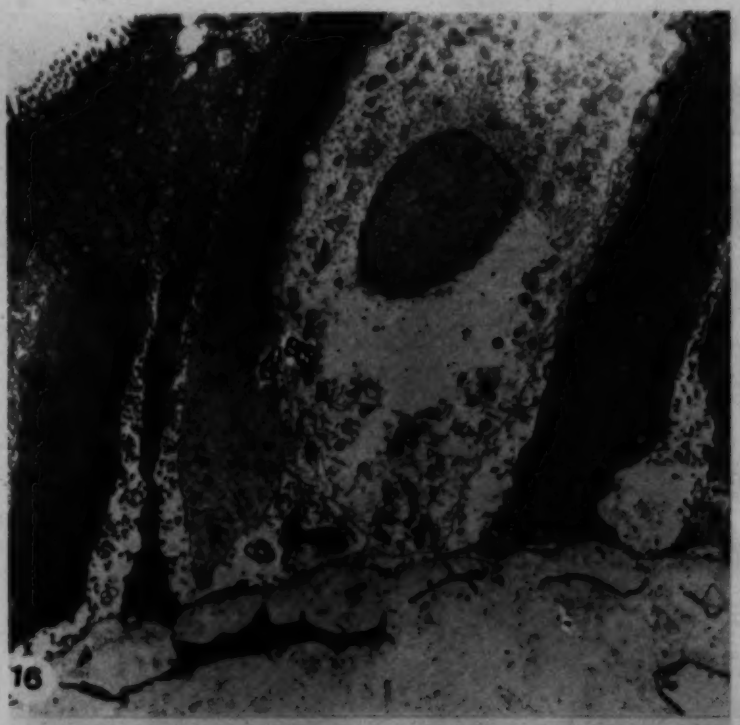
15 b

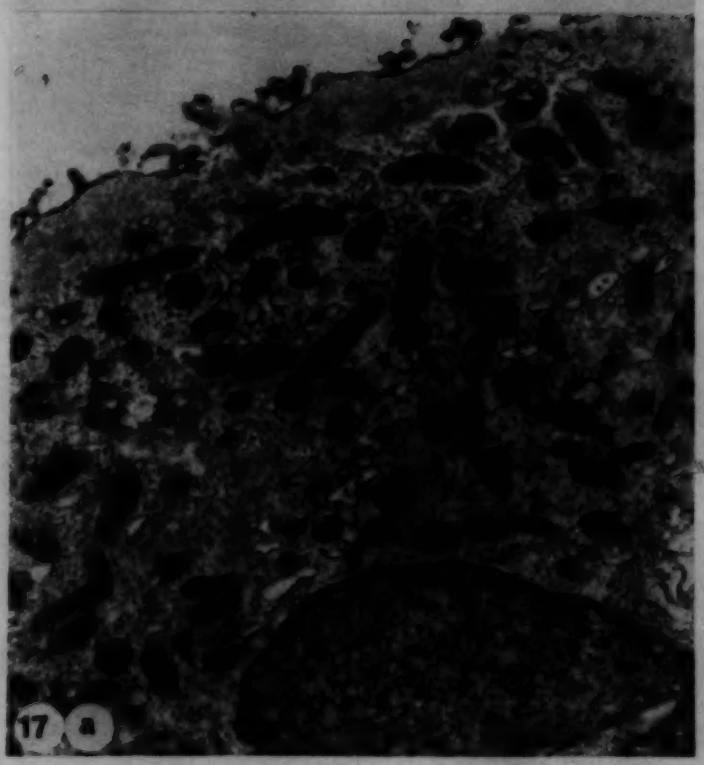
FIGURE 16: Transmission electron micrograph of gallbladder epithelium at day 3 showing an edematous cell in the epithelial sheet containing widely dispersed organelles in its cytoplasmic matrix.

x2300

FIGURE 17a: Transmission electron micrograph of gallbladder epithelium at day 3 showing shortening and partial destruction of the apical microvilli; the cristae of the mitochondria are indistinct and the endoplasmic reticulum is dilated.

x2500


16


17 a

These features were absent from epithelial cells that had many mucus vacuoles and dilated smooth endoplasmic reticulum but normal-appearing mitochondria. These cytoplasmic changes were observed in most of the specimens examined at later stages. No inflammatory cells were seen.

At days 2 and 3, cellular degeneration and exfoliation were more severe. In some specimens, granular endoplasmic reticulum was adherent to granular electron-dense bodies (Fig 18). The organelles had lost some of the ribosomes which normally attach to their surface.

At day 4, the most conspicuous feature was increased frequency of dilated lateral intercellular spaces (Fig. 19a,b). Tight junctions remained intact near the luminal aspect of these interfaces, and the spaces widened progressively towards the basement membrane. Adjacent cells appeared compressed laterally so that their cytoplasm appeared darker than usual and the organelles fainter, but mucus droplets were apparent. The basal lamina was markedly infolded and the underlying lamina propria appeared edematous (Fig. 19c). The mucosal venules and capillaries were very dilated and their walls thinned, but no structural abnormalities were noted in the endothelial cells.

At day 5, large areas of cellular disruption were observed and the gallbladder lumen contained cellular debris. In some parts of the mucosa, only remnants of the cytoplasmic organelles were visible at sites originally occupied by surface cells (Fig. 20). In other instances, remnants of cell membranes remained attached to neighboring cells and basement membrane, creating the impression of a cell whose apical membrane had ruptured and allowed extrusion of the cytoplasmic contents into the lumen of the gallbladder.

FIGURE 17b: Transmission electron micrograph of gallbladder epithelium at day 3 demonstrating blebbing of the apical plasma membrane as well as alterations, loss of structural details and the presence of inclusion in the mitochondria, the endoplasmic reticulum is dilated.

x2500

FIGURE 18: Transmission electron micrograph of an epithelial cell at day 3 showing granular endoplasmic reticulum surrounding an electron-dense substance.

x2300

17 b

18

FIGURE 19: Transmission electron micrograph of gallbladder epithelium 4 days after ingestion of the lithogenic diet showing alterations in the spatial organization of the epithelial sheet.

Figure 19a. The lateral intercellular spaces are dilated towards the basal part of the cells; mucus vacuoles are present mostly in the supranuclear regions of the cytoplasm.

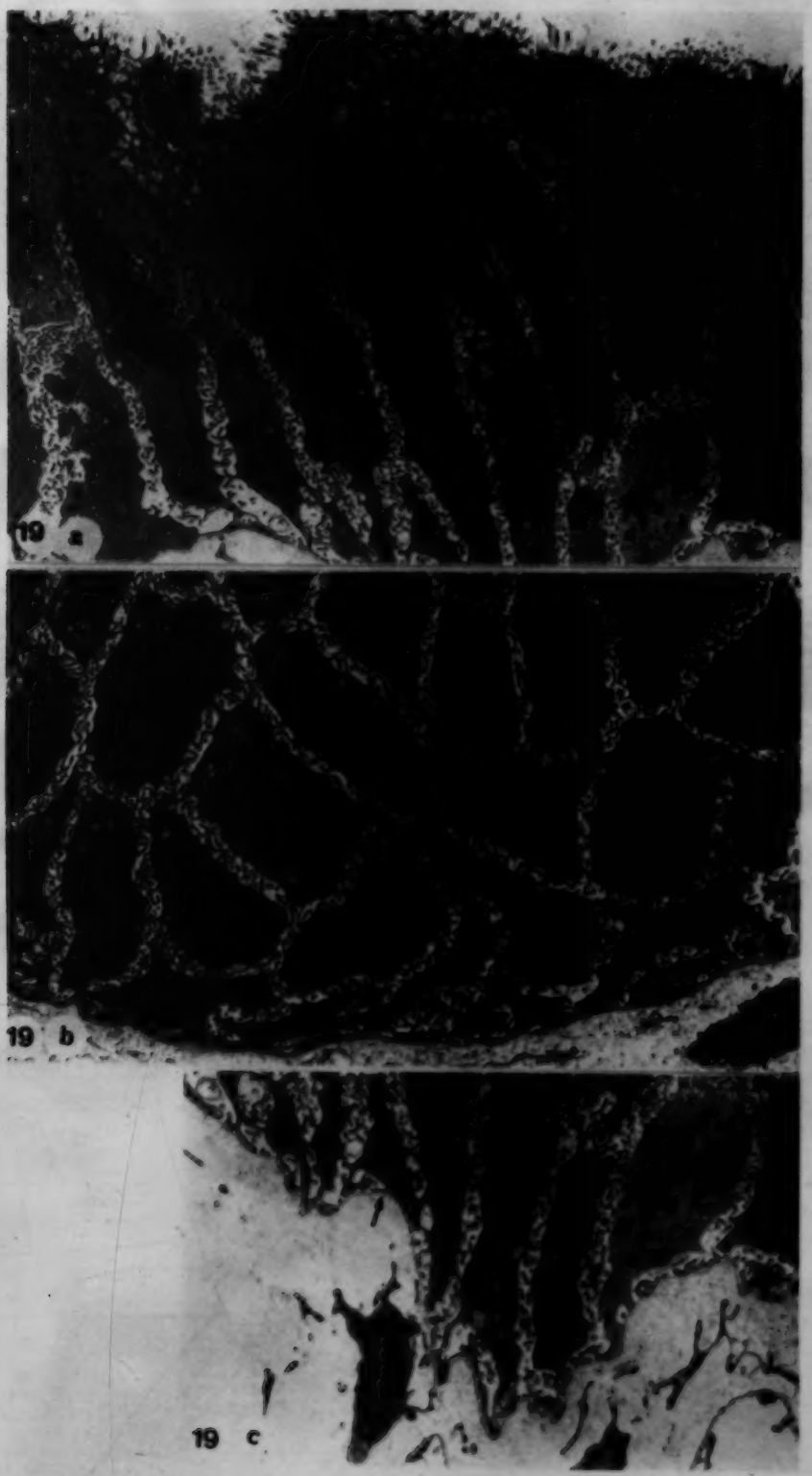
x2300

Figure 19b. The prominence of the dilated lateral intercellular spaces in transverse section of the epithelial sheet.

x2300

Figure 19c. The basal lamina (arrow) is markedly folded and the underlying lamina propria is edematous.

x2300


19 a
19 b
19 c

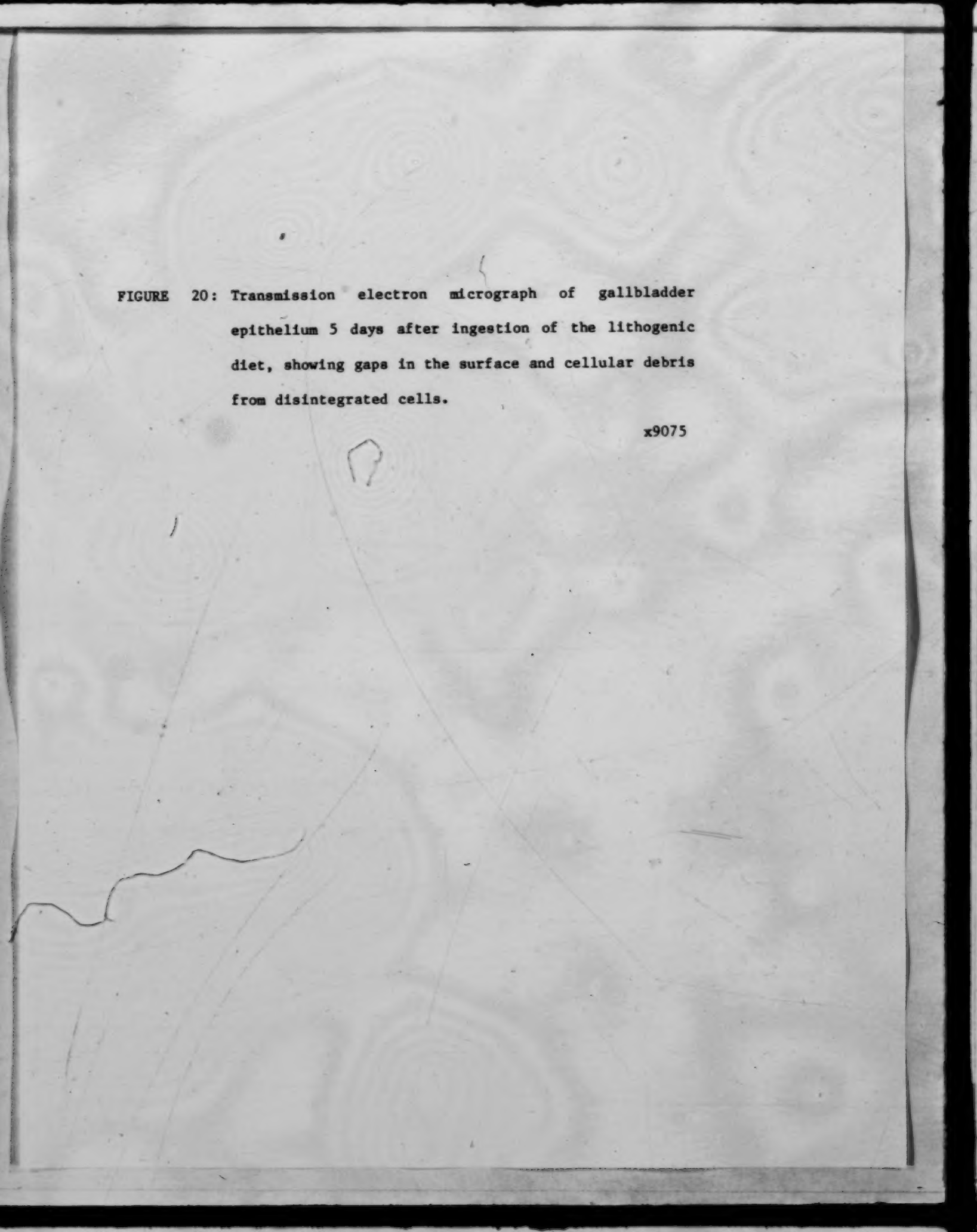
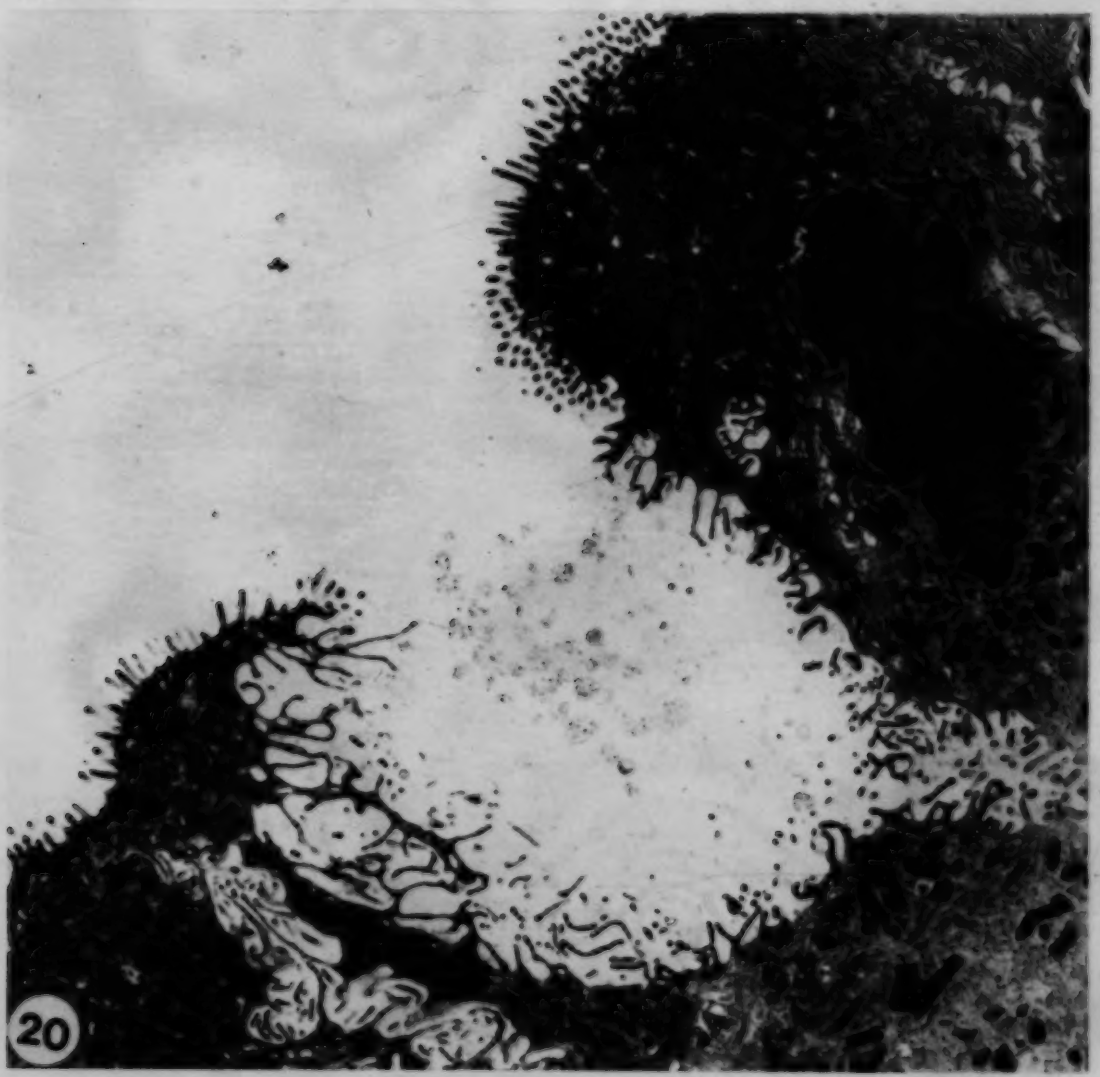


FIGURE 20: Transmission electron micrograph of gallbladder epithelium 5 days after ingestion of the lithogenic diet, showing gaps in the surface and cellular debris from disintegrated cells.

x9075


20

DISCUSSION

Rabbits fed a DHC-rich diet consistently develop gallstones, which are composed largely of glycine-conjugated bile acids (105); the stones form within 9 to 10 days (82). The findings in the present study and others (76, 82) clearly demonstrate that the gallbladder epithelium undergoes subtle changes long before gallstone formation. The structural alteration was rapid, beginning within 24 hours of the start of the lithogenic diet; it consisted of cellular exfoliation, deposition of osmiophilic droplets between the epithelial cells and basal lamina as well as in foam cells of the lamina propria, and mucus hypersecretion. Furthermore, TEM studies showed that these cellular alterations were associated with cytologic changes, including dilation of both the endoplasmic reticulum and mitochondria. Thus, at least in this model, the gallbladder epithelium is injured very early during lithogenesis. In these studies, however, unlike those studies by Mosbach and Bevans (69), also in rabbits, inflammatory-cell infiltration was not prominent.

The ultrastructural response of the gallbladder epithelium in the present study can be considered in three phases. In the early phase (within 24 hours of the start of the diet) changes in the intracellular morphology of the epithelial cells predominate. In causing its cytolytic effect the offending agent may enter the cell by diffusion or pinocytosis, both of which are known to occur in the rabbit gallbladder (17). The smooth endoplasmic reticulum bears many complex enzyme

systems on its surface that are capable of degrading most exogenous noxious substance(106). Thus the distension of the organelle in this phase is regarded as an adaptive response by the epithelial cell which increases its ability to detoxify the irritant. Furthermore, the epithelial cells respond to the intracellular insult by increasing their production of mucous glycoprotein (107). In this phase, ultrastructural changes involving the microvilli, plasma membranes, or tight junctions are not prominent features.

The consequence of epithelial-cell membrane dysfunction were manifest in the second phase (days 2 to 4). Cells that had undergone intensive internal disruption exhibited pale-staining cytoplasms and widely dispersed organelles, indicative of intracellular edema (106). These injured cells show two patterns of structural changes in the apical plasma membrane. First, there is destruction of the microvilli, leaving only short, stubby knobs at the luminal surface of the cells. The second change consisted of formation of blebs of cytoplasm at the cell surface. These changes were mostly associated with cells that exhibited swelling of the endoplasmic reticulum and mitochondria.

The third phase, day 5 and was characterized by increased shedding of damaged cells with cellular debris into the gallbladder lumen. These findings are well demonstrated on scanning EM. Indeed, the author is not aware of any other study that has shown gallbladder epithelial injury on scanning EM during experimental cholelithiasis. The combined cytologic and cell-membrane effect of the offending agent culminated in more extensive cellular disruption. Cells at the tip and upper third of the mucosal folds appeared to be least able to withstand the adverse effect of irritants, being older (27) and more engaged in fluid

transport (17). This may well explain the present finding of greater cellular disruption at these sites than lower down the folds.

Although the present study did not examine the cause of the injury to the gallbladder epithelium, the lack of correlation between gallbladder luminal volume and duration of the diet clearly precludes distension of the organ as a cause in this model. These findings differed from those reported by Hall et al (92), who found increased hepatic bile flow and enlargement of the gallbladder in rabbits fed a Borgstrom diet (40% casein and 15% olive oil) for 8-12 months. They postulated that distension of the gallbladder could cause transient ischemia of the mucosa, thereby damaging it. The possibility that bile acids are the irritants (76) was supported by Lee and Scott (72), who demonstrated in rabbits fed DHC that gallbladder epithelial injury coincided with an increased biliary concentration of allodeoxycholate. They attributed the observed effects to the toxic action of bile acids on cell membranes, organellar membranes and their enzymes. Histochemical studies with light and electron microscopy would be necessary to demonstrate concurrent changes in lysosomal enzyme activity and hence their role in epithelial cell-injury in this model. Studies of human gallbladder have implicated lysosomal enzymes released from damaged mucosa in the pathogenesis of acute cholecystitis (108). Phospholipase A, a lysosomal enzyme in the gallbladder epithelium, converts lecithin to lysolecithin, and the latter causes further mucosal injury. In rabbit gallbladder in vitro, lysolethicin damages epithelial cells, causing mucosal edema and increased membrane permeability and resulting in the release of acid phosphatase into the gallbladder lumen (109).

One of the most distinctive findings of this study consisted of

large osmiophilic droplets between the epithelial cells and the basal lamina as well as in foam cells in the lamina propria. Although the nature of the droplets is not known from the present study, their staining characteristics indicated that their contents were lipid. The microscopic appearance of the gallbladders here, resembles those from dogs fed a cholesterol-cholic acid diet (110) and is also similar to the picture of human gallbladder with cholesterolosis (111,112,113), however, in the latter, the lipid droplets are present in foam cells only. The pathogenesis of this microscopic finding and its role, if any, in cholelithiasis in this model is not known and can only be speculated at this juncture. The epithelial injury may alter the lipid metabolism or transport, leading to its accummulation in the gallbladder wall.

The findings on histochemistry show that specimens from experimental animals stained more intensely for mucus than those from the control group. The epithelium was producing more acidic mucus at this stage than the neutral. Although there was no quantitative assessment of mucus production (eg. morphometric measurement or determination of the amount of hexose amines), the intensity of the mucus stain and the increased subapical granules on TEM suggest mucus hypersecretion. Several groups of investigators, studying various animal species, have called attention to the increased production of mucous glycoprotein by the gallbladder epithelium during experimental lithogenesis (50,76-78,82). They stressed that this precedes formation of gallstones and thus mucus may be a specific nucleation factor (50). Gallstone formation is a complex process, involving more than simple precipitation of bile constituents. It is generally accepted that nidus

formation, and the retention of particles in the gallbladder and their growth into macroscopic stones, are also important stages (103). In the rabbit model of cholelithiasis, stones are formed by the precipitation of calcium glyco-allodeoxycholic acid (105). In this situation, mucus may facilitate gallstone formation by binding the precipitated bile salts into macrocrystals and its visco-elastic property would tend to prevent evacuation of these crystals (82).

CONCLUSIONS

A study of this type is limited in its extrapolation to cholesterol gallstone disease in humans for reasons outlined previously (page 23). Furthermore, it might be argued that the epithelial changes observed represented cells in various stages of degeneration as a normal process of aging and not due to effects of the experimental diet. Although this possibility cannot be ruled out, correlation of the findings in large number of TEM samples with those on SEM and light microscopy makes normal degenerative changes unlikely.

The significance of the present study lies in its demonstration of epithelial cell-injury long before gallstones are formed in this model. Osmiophilic lipid droplets were deposited in the gallbladder wall before any stones were formed. However, the role of this lipid deposition, if any, in the present model of cholelithiasis remains to be established.

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